

PATENT ABSTRACTS OF JAPAN

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(54) ALFA-L-RHAMNOSIDASE AND ITS PRODUCTION

(57)Abstract:

PROBLEM TO BE SOLVED: To obtain a new  $\alpha$ -L-rhamnosidase originated from a microorganism of the genus *Pichia*, having activity to release p-nitrophenol by using p-nitrophenyl- $\alpha$ -L-rhamnopyranoside as a substrate, having glucose tolerance, useful for wine fermentation or the like.

SOLUTION: This new  $\alpha$ -L-rhamnosidase has the following features: p-nitrophenol is released by acting it on p-nitrophenyl- $\alpha$ -L-rhamnopyranoside as a substrate; when the substrate is made to react for 10 min at 30°C, the optimum pH is 6.0-7.0; when held for 30 min at 30°C, the  $\alpha$ -L-rhamnosidase exhibits stable enzymatic activity at pH 5.0-7.0; when the substrate is made to react for 10 min at pH 6.5, the optimum temperature is about 40°C; when held for 30 min at pH 6.5, the  $\alpha$ -L-rhamnosidase is stable up to 20°C and gradually deactivated with the exceeding of the temperature; the molecular weight is about 88,000 daltons (SDS-PAGE); and the  $\alpha$ -L-rhamnosidase has glucose tolerance and ethanol tolerance. This enzyme increases fragrance components in wine fermentation, and it is useful for improving the quality of wine or the like. The enzyme is obtained from the fermentation product of a microorganism of the genus *Pichia*.

CLAIMS

[Claim(s)]

[Claim 1] Alpha-L-rhamnosidase which has the following character.

- (1) OPERATION -- when p-nitrophenyl alpha-L-rhamnopyranoside is made to act as a substrate, separate p-nitrophenol.
- (2) Optimal pH : optimal pH at a time of using p-nitrophenyl alpha-L-rhamnopyranoside as a substrate and making it react for 10 minutes at 30 °C is in the 6.0 to 7.0 neighborhood.
- (3) pH stability : when it holds for 30 minutes at 30 °C, enzyme activity stable in pH 5.0-7.0 is shown.
- (4) Optimum temperature : optimum temperature at a time of using p-nitrophenyl alpha-L-rhamnopyranoside as a substrate and making it react for 10 minutes at 30 °C pH 6.5 is about 40 °C.
- (5) Thermal stability : when it holds for 30 minutes in pH 6.5, if enzyme activity stable to 20 °C is shown and it is exceeded, it will be deactivated gradually.
- (6) Inhibitor : mercury para-chlorobenzoate, mercuric chloride, and a cupric chloride receive inhibition, and it is hardly prevented in ethylenediaminetetraacetic acid and mercaptoethanol.

(7) A molecular weight : a molecular weight measured by an SDS-polyacrylamide-gel-electrophoresis method is about 88,000 dalton.

(8) An isoelectric point: An isoelectric point by isoelectric focusing is pI4.9.

(9) Glucose tolerance : 76% of relative activity is shown in 500mM glucose.

(10) Ethanol resistance : 67% of relative activity is shown in 20 capacity % ethanol.

[Claim 2]A manufacturing method of alpha-L-rhamnosidase extracting the alpha-L-rhamnosidase according to claim 1 from a culture produced by cultivating a microorganism belonging to the Pichia (Pichia) group.

[Claim 3]A manufacturing method of alpha-L-rhamnosidase extracting the alpha-L-rhamnosidase according to claim 1 from a culture produced by cultivating a microorganism belonging to the Pichia (Pichia) group by a culture medium containing rhamnose (rhamnose).

[Claim 4]The Pichia ANGUSUTA (Pichia angusta)X349 share which has the alpha-L-rhamnosidase production ability according to claim 1 (FERM P-17211).

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## DETAILED DESCRIPTION

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[Detailed Description of the Invention]

[0001]

[Field of the Invention]This invention relates to the microorganism which produces new alpha-L-rhamnosidase, its manufacturing method, and its enzyme.

[0002]

[Description of the Prior Art]Linalool (linalool), geraniol (geraniol) which are contained in a grape, Monoterpenes, such as Nellore (nerol), citronellol (citronelol), and the alpha-terpineol (terpineol), have contributed to the scent of a grape or wine greatly. Although such monoterpenes are contained also as fragrant educt, the many exist as a glycoside which does not have fragrance.

[0003]Many of these glycosides 6-O-alpha-L-arabinofuranosyl beta-D-glucopyranoside (6-O-alpha-L-arabinofuranosyl-beta-D-glucopyranosides), 6-O-alpha-L-rhamnopyranosyl beta-D-glucopyranoside (6-O-alpha-L-rhamnopyranosyl-beta-D-glucopyranosides), It is known that they are jig RIKOSHIDO called 6-O-beta-D-apiofuranosyl beta-D-glucopyranoside (6-O-beta-D-apiofuranosyl-beta-D-glucopyranosides).

[0004]If the hydrolysis reaction of these jig RIKOSHIDO is carried out enzymatically, fragrant monoterpenes will separate. This hydrolysis reaction is performed in two steps. First Namely, L-rhamnosidase (alpha-L-rhamnosidase), Alpha-L-arabino flannel SHIDAZE (alpha-L-arabinofuranosidase), A glycosidic linkage is cut by beta-APIOSHIDAZE (beta-apiosidase) etc. and mono- \*\*\*\*\*- beta-D-glucoside (monoterpenyl-beta-D-glucosides) corresponding, respectively is produced. Subsequently, beta-D-glucosidase (beta-D-glucosidase) acts on this mono- \*\*\*\*\*- beta-D-glucoside. Alpha-L-rhamnosidase is an enzyme which hydrolyzes the alpha-L-rhamnopyranoside residue of the nonreducing terminal of the polysaccharide containing alpha-L-rhamnose (L-rhamnose) among the hydrolase which carries out the catalyst of the first-step reaction. Therefore, by using alpha-L-rhamnosidase with beta-D-glucosidase etc., a perfume component can be made to be able to increase in wine brewing, and the quality of wine can be raised.

[0005]However, in using these enzymes for wine brewing, there is a problem which many should conquer. It is that it is required for one to have enzyme activity also under

existence of glucose or ethanol. In the case of the enzyme of bacteria origin, by a biomass external secretion [ of that there is a good rare colander bacteria smell and mold origin ] type crude enzyme, there is a problem that a good rare colander side reaction occurs for other catalytic activity in one. Although there is a report about these bacteria or alpha-L-rhamnosidase of mold origin, on the other hand, there is no report about the thing of yeast origin. If it says strictly, the thing of *Saccharomyces cerevisiae* (*Saccharomyces cerevisiae*) origin is reported, but the main catalytic activity is beta-D-glucosidase and is only also having alpha-L-rhamnosidase activity slightly.

[0006]

[Problem(s) to be Solved by the Invention]Therefore, the purpose of this invention is to provide the microorganism which produces new alpha-L-rhamnosidase provided with the characteristic which can be used for wine brewing, its manufacturing method, and its enzyme.

[0007]

[Means for Solving the Problem]This invention persons ask for a microorganism which can produce alpha-L-rhamnosidase provided with the characteristic which can be used for wine brewing, As a result of searching centering on yeast with until now few examples of a report especially, one strain belonging to the *Pichia* (*Pichia*) group finds out producing new alpha-L-rhamnosidase, and came to complete this invention. Alpha-L-rhamnosidase activity is measured by a measuring method which crushes a biomass and for which a centrifugal supernatant liquid fraction is later mentioned after culture by a culture medium which specifically makes L-rhamnose the only carbon source for many microorganisms isolated from various kinds of sources of separation, and the target microorganism is chosen.

[0008]That is, according to this invention, alpha-L-rhamnosidase having the following character is provided.

(1) OPERATION -- when p-nitrophenyl alpha-L-rhamnopyranoside is made to act as a substrate, separate p-nitrophenol.

(2) Optimal pH : optimal pH at a time of using p-nitrophenyl alpha-L-rhamnopyranoside as a substrate and making it react for 10 minutes at 30 °C is in the 5.0 to 7.0 neighborhood.

(3) pH stability : when it holds for 30 minutes at 30 °C, enzyme activity stable in pH 5.0-7.0 is shown.

(4) Optimum temperature : optimum temperature at a time of using p-nitrophenyl alpha-L-rhamnopyranoside as a substrate and making it react for 10 minutes at 30 °C pH 6.5 is about 40 °C.

(5) Thermal stability : when it holds for 30 minutes in pH6.5, if enzyme activity stable to 20 °C is shown and it is exceeded, it will be deactivated gradually.

[0009](6) Inhibitor : mercury para-chlorobenzoate, mercuric chloride, and a cupric chloride receive inhibition, and it is hardly prevented in ethylenediaminetetraacetic acid and mercaptoethanol.

(7) Molecular weight : a molecular weight measured by an SDS-polyacrylamide-gel-electrophoresis method is about 88,000 dalton.

(8) Isoelectric point: An isoelectric point by isoelectric focusing is pI4.9.

(9) Glucose tolerance : 76% of relative activity is shown in 500mM glucose.

(10) Ethanol resistance : 67% of relative activity is shown in 20 capacity % ethanol.

[0010]A manufacturing method of alpha-L-rhamnosidase of this invention is the method of acquiring alpha-L-rhamnosidase from culture medium obtained by belonging to the

*Pichia* (*Phicia*) group and cultivating a microorganism which produces alpha-L-rhamnosidase.

[0011]Character of a separation strain to produce alpha-L-rhamnosidase of this invention is shown below.

A. Form a colony of morphometrical characteristic 1 opalescence.

2) Germination performs asexual growth.

3) Form 1 thru/ or 4 ascospores of a bowler hat form by a glucose malt culture medium.

[0012]

B. physiological character 1 fermentability Glucose + galactose . - malt sugar - sucrose - trehalose . + melibiose - lactose -2 utilization nature . glucose + lactose - galactose . - raffinose - sorbose - ribitol . + ribose + mannitol + xylose + inositol - rhamnose + methanol + sucrose + ethanol + malt sugar + nitrate + melibiose - (+utilization carries out -utilization does not carry out)

[0013]It refers to BARNETT & PAYNE YARROW YEASTS:Characteristics and identification (Second edition) based on the above mycology character, This strain was identified to *Pichia ANGUSUTA* (*Pichia angusta*). It was judged that it was a new strain from a point of producing above-mentioned new alpha-L-rhamnosidase. Therefore, it was named *Pichia ANGUSUTA* (*Pichia angusta*) X349, and a bacteria stock was deposited with National Institute of Bioscience and Human Technology. A deposition number is FERM P-17211. If a microorganism used for this invention is a strain which produces alpha-L-rhamnosidase of this invention by a microorganism belonging to a *Pichia* group besides said *Pichia ANGUSUTA* (*Pichia angusta*) X349, it will not interfere.

[0014]In this invention, it may be used for culture of the usual microorganism as a culture medium which produces alpha-L-rhamnosidase, and especially if a microorganism used grows, it will not be restricted. As a carbon source, although sucrose etc. can be used, for example, a point of producing a lot of alpha-L-rhamnosidase to especially L-rhamnose is desirable. As a nitrogen source, peptone is raised, for example. To a culture medium, mineral salt, for example, sodium chloride, besides a carbon source and a nitrogen source can be added. Although the culture temperature should just be a temperature which a microorganism to be used grows, near [ good ] 28 \*\* of growth is preferred. Initiation pH of a culture medium to be used has the 4.0 preferred neighborhoods. Alpha-L-rhamnosidase is accumulated into culture medium at culture time by [ for growth of the bacillus concerned, and alpha-L-rhamnosidase production / sufficient ] usually cultivating for 48 hours, although time continuation is carried out.

[0015]Biomasses are collected with filtration, centrifugal separation, or filtration after culture, and supernatant liquid produced by crushing a biomass by mechanical disruption processing of lytic enzyme processing or ultrasonication, an French press, a homogenizer, etc., and centrifuging is used as crude enzyme liquid. Although crude enzyme liquid can also be used as it is, separation refinement can be carried out according to the separation refinement methods of common enzymes, such as ammonium sulfate curing salting and a solvent sedimentation method. An enzyme preparation with high activity purity can also be prepared combining suitably a refining means of the usual enzymes, such as ion exchange chromatography and gel filtration chromatography. The character of this invention enzyme obtained by refining combining various chromatography methods from crude enzyme liquid is as follows. Measurement of enzyme activity was performed to below by a following method.

[0016][Enzyme activity measurement] 0.2 ml of 2mM p-nitrophenyl alpha-L-rhamnopyranoside solutions which dissolved in the buffer solution are mixed to 0.2 ml of enzyme sample solutions diluted with 50mM sodium phosphate buffer solution (pH 6.5), and it is made to react to them for 10 minutes at 30 \*\*. Then, 1.6 ml of 1M sodium carbonate is added, a reaction is stopped, and p-nitrophenol separated by measuring an absorbance at 405 nm is quantified. One enzyme unit (U) which shows enzyme activity was defined in the above-mentioned conditions, i.e., 30 \*\*, and pH 6.5 as an amount of enzymes which makes p-nitrophenol of 1micromol generate in 1 minute after p-nitrophenyl alpha-L-rhamnopyranoside.

[0017](1) OPERATION -- when p-nitrophenyl alpha-L-rhamnopyranoside is made to act as a substrate, separate p-nitrophenol.

Optimal pH : (2) p-nitrophenyl alpha-L-rhamnopyranoside is used as a substrate, In accordance with said enzyme activity measurement, relative activity was measured except using a 50mM citrate-phosphate buffer solution (pH 3.0-7.0), 50mM phosphate buffer solution (pH 8.0-9.0), and 50mM glycine sodium hydroxide buffer solution (pH 10.0) as buffer solution. The result is as being shown in drawing 1, and reaction optimal pH is in the 6.0 to 7.0 neighborhood.

pH stability : (3) A 50mM citrate-phosphate buffer solution (pH 3.0-7.0), After holding for 30 minutes at 30 \*\* in each buffer solution of 50mM phosphate buffer solution (pH 8.0-9.0) and 50mM glycine sodium hydroxide buffer solution (pH 10.0), residual activity was measured in accordance with said enzyme activity measurement. The result is as being shown in drawing 1, and a stable pH range is pH 5.0-7.0.

(4) Optimum temperature : in accordance with said enzyme activity measurement, relative activity was measured at various temperature. The result is as being shown in drawing 2, and optimum temperature is about 40 \*\*.

(5) Thermal stability : after holding for 30 minutes in 50mM phosphate buffer solution (pH 6.5), residual activity was measured in accordance with said enzyme activity measurement. As a result, if this enzyme shows enzyme activity stable to 20 \*\* and it is exceeded as shown in drawing 2, it will be deactivated gradually.

[0018](6) Influence of various reagents : influence on this enzyme of various reagents was investigated. That is, various reagents shown in Table 1 were dissolved so that it might become 50mM phosphate buffer solution (pH 6.5) with 1mM, an enzyme solution was mixed to this, residual enzyme activity after holding for 10 minutes in 30 \*\* was measured in accordance with said enzyme activity measurement, and a result of Table 1 was obtained.

[Table 1]

[0019](7) Molecular weight : it is SDS-polyacrylamide gel electrophoresis () about this refined enzyme. [ PhastSystem and ] A molecular weight which measured by performing PhastGel homogeneous12.5 and PhastGel SDS bufferstrips (Pharmacia manufacture) is about 88,000 dalton. To a standard of a molecular weight, b type phosphorylase (molecular weight of 94,000 dalton), Bovine serum albumin (molecular weight of 67,000 dalton), ovalbumin (molecular weight of 43,000 dalton), Carbonic anhydro RAZE (molecular weight of 30,000 dalton), soybean typsin inhibitor (molecular weight of 20,100 dalton), and lactalbumin (molecular weight of 14,400 dalton) were used.

(8) Isoelectric point: The isoelectric point which measured about this refined enzyme by performing isoelectric focusing (PhastSystem, PhastGel IEF 3-9 (Pharmacia manufacture)) is pI4.9. To the standard of an isoelectric point, horse myoglobin (acidic:pI6.85), Homo sapiens carbonic anhydro RAZE (pI6.55), cow carbonic anhydro

RAZEB (pI5.85), the beta-lactoglobulin A (pI5.20), a soybean trypsin inhibitor (pI4.55), and amyloglucosidase (pI3.50) were used.

[0020](9) Glucose tolerance : in accordance with said enzyme activity measurement, relative activity was measured in 50mM phosphate buffer solution (pH 6.5) containing glucose of 0 - 500mM concentration. The result is as being shown in drawing 3, and shows 76% of relative activity also in 500mM glucose.

(10) Ethanol resistance : in accordance with said enzyme activity measurement, relative activity was measured in 50mM phosphate buffer solution (pH 6.5) containing the ethanol of 0 - 20 capacity % concentration. It is as being shown in drawing 4, and in 10 capacity % ethanol, the result shows 85% of relative activity, and shows 67% of relative activity in 20 capacity % ethanol.

[0021]

[Example]Next, although the example of this invention is given and this invention is explained in detail, the example shown below is an example and does not limit the technical scope of this invention.

Example 1 : Culture of a strain, and 1.0% of production L-rhamnose of alpha-L-rhamnosidase, Peptone 0.5%, the culture medium which consists of 0.3% of a yeast extract and 0.3% of a malt extract was adjusted the pH to 4.0 with chloride, 100 ml of this was taught to each 40 Erlenmeyer flasks of 500-ml \*\*, and autoclaving was carried out for 15 minutes 120 \*\*. The *Pichia ANGUSUTA* (*Pichia angusta*)X349 share was inoculated into this, and it cultivated on the rotation agitator for two days at 28 \*\* and 220 rpm. The obtained culture medium 4L is centrifuged (for 1000xg and 10 minutes), biomasses are collected, and it is 20mM. A screw/tris buffers (pH 6.5) washed twice. This was suspended to the buffer solution, the 2.5-times the amount glass bead (0.45-0.55 mm in diameter) of biomass weight was added, and the biomass was crushed. Except for biomass debris, the cell extract was obtained by centrifugal separation (for 1000xg and 10 minutes). Ultracentrifuge processing (for 100,000xg and 90 minutes) of this was carried out, and it separated into the microsome fraction and the supernatant liquid fraction. Since all the activity existed in the supernatant liquid fraction when such fraction alpha-L-rhamnosidase activity was measured, the refining operation described below was presented with the supernatant liquid fraction.

[0022]Example 2: Especially refining operation shown below in refining of an enzyme was altogether performed at 4 \*\*, unless it refused.

(1) Ammonium sulfate precipitate refined 600 ml of supernatant liquid fractions obtained in ammonium sulfate precipitate example 1. the precipitate formed from saturation in the fraction of saturation 80% 30% -- centrifugal separation (1,000xg.) It carried out for 30 minutes, collected, dissolved in the 100mM sodium acetate buffer solution (pH 6.0) containing 1M NaCl, 1mM MnCl<sub>2</sub>, 1mM CaCl<sub>2</sub>, and 0.003mM NaN<sub>3</sub>, and dialyzed to the buffer solution.

(2) The enzyme solution which carried out concanavalin A-sepharose column chromatography dialysis was given to the concanavalin A-sepharose column (phi2.6x6.5cm) beforehand equilibrated with the buffer solution. Since the target enzyme did not stick to this column, it was eluted with the buffer solution. Activity fractions are collected, and it condenses with ultrafiltration equipment (made by PM-10:Amicon), and is 20mM. It dialyzed to a screw/tris buffers (pH 6.5).

(3) DEAE Bio-Gel A They are 20mM beforehand about the enzyme solution which carried out agarose column chromatography dialysis. The DEAE Bio-Gel A agarose column (phi2.6x6.0cm) equilibrated with a screw/tris buffers (pH 6.5) was given. After

the buffer solution washed, it was eluted by the sodium chloride linear gradient (0-0.3M) using the buffer solution. Activity fractions were collected and it dialyzed to 50mM sodium acetate buffer solution (pH 5.0).

[0023](4) An arabinose sepharose 6B affinity column chromatography rhamnose sepharose 6B affinity carrier, In accordance with how to recommend a manufacturing company for the epoxy activation sepharose 6B (made by Pharmacia biotech AB), rhamnose was combined and it prepared. The enzyme liquid obtained by the DEAE Bio-GelA agarose column chromatography mentioned above, The rhamnose sepharose 6B affinity column (phi1.5x7.2cm) beforehand equilibrated with 50mM sodium acetate buffer solution (pH 5.0) was given, and the buffer solution washed. Next, it was eluted with the buffer solution containing 1M NaCl, activity fractions were collected, and it dialyzed to distilled water.

(5) The enzyme solution which carried out hydroxyapatite column chromatography dialysis, The hydroxyapatite column (phi1.5x2.8cm) beforehand equilibrated with 10mM sodium phosphate buffer solution was given, the buffer solution washed enough, the back was eluted by the sodium phosphate linear gradient (10 - 200mM), and activity fractions were collected.

[0024]The activity of the enzyme in every above purification process is shown in Table 2. Cow serum albumin is used for protein as a standard substance, and it is the bottom in fixed quantity by the BURADOFODO method (Bradfordmethod).

[Table 2]

rha-sepharose 6B: Rhamnose sepharose 6B[0025]Example 3: The decomposition examination of the various chromophoric substrates shown in Table 3 was done using the refining enzymes obtained in substrate specificity example 2. The decomposition examination was done according to the method of said enzyme activity measurement, and the existence of activity was investigated by generation of p-nitrophenol. A result is shown in Table 3.

[Table 3]

[0026]Although this enzyme acts on p-nitrophenyl alpha-L-rhamnopyranoside and p-nitrophenol is separated, It hardly acts on p-nitrophenyl alpha-L-arabino furanoside, p-nitrophenyl beta-D-glucopyranoside, p-nitrophenyl beta-D-xylopyranoside, and p-nitrophenyl beta-D-cello BIOSHIDO, but it turns out that it is an enzyme with high substrate specificity.

[0027]1000 ml of grape juice (muscat OBU Alexandria kind) adjusted the pH to 7.0 by operation sodium hydroxide to the glycosides of Example 4:grape juice origin -- a synthetic adsorption resin column (phi -- 22 mm x 350 mm) Amberlite XAD-2: It let it pass to made in loam and Haas. 500 ml of distilled water washed this, and water soluble compounds were removed. After removing through and free terpenes for 1000 ml of pentane-ether (1:1) furthermore, glycosides were made eluted with 1000 ml of methanol. Concentration hardening by drying of this fraction was carried out under 30 \*\* and decompression, and 100 mg of glycosides were obtained. 10 mg of the above-mentioned glycosides were dissolved in 2 ml (0.5U, 50mM sodium phosphate buffer solution (pH 6.5)) of refining enzyme solutions obtained in Example 2, and it was made to react for two days at 30 \*\*. After 5 ml of pentane-ether (1:1) having extracted the separated terpenes twice and condensing it, it analyzed on condition of the following using capillary gas chromatography. A result is shown in Table 4. Thus, the enzyme of this invention disassembles the glycosides of grape juice, and has the outstanding character to which a perfume component is made to increase notably.

[Table 4]

[0028](Analysis condition)

device: -- Hewlett Packard 5980 column: -- a DB-WAX capillary column (made by phi0.25-mm x 30 m: J&W Scientific)

detector: -- FID helium flow \*\*: -- a part for 2.0 ml / -- temperature program: -- -> with a temperature up of 75 \*\* 220 \*\* (a part for 4 \*\*), and 220 \*\* (15 minutes) of maintenance

sample injection section temperature: -- 230 \*\* primary detecting element temperature:

-- 220 \*\* internal standard: -- amount of 2-ethyl-1-hexanol samples: -- 2 microL

[0029] Example 5: Have a rhamnosyl group in the operation nonreducing terminal to the rhamnosyl glycoside of natural flavonoid. The decomposition product of naringin (naringin), rutin (rutin), hesperidin (hesperidin), and the rhamnosyl glycoside of quercitrin (quercitrin) was analyzed using silica gel thin layer chromatography. That is, the reaction solution in which 0.5 ml of solutions (0.5U, 50mM sodium phosphate buffer solution pH6.5) of the refining enzymes obtained in Example 2 were made to dissolve 5 mg of substrates was held at 30 \*\* overnight. This sample was given to 4.0 microL and a silica gel thin layer (DC-Alufolien Kieselgel 60: made by Merck Co.). After developing using acetonitrile distilled water (5:1, V/V) mixture, using rhamnose and glucose as a standard substance, the ethanol solution of sulfuric acid was sprayed 10%, it heated for 5 minutes at 105 \*\*, and sugars were detected.

[0030] As a result, naringin (naringin), the rutin (rutin), and the rhamnosyl glycoside of hesperidin (hesperidin) received decomposition by alpha-L-rhamnosidase, and rhamnose was detected. On the other hand, rhamnose was not detected about the rhamnosyl glycoside of quercitrin (quercitrin).

[0031]

[Effect of the Invention] this invention enzyme has the activity which was dramatically excellent under high-concentration glucose and a high-concentration ethanol existence. It can use for brewing of wine.

That is, by using this invention enzyme with beta-D-glucosidase etc., a perfume component can be made to be able to increase in wine brewing, and the quality of wine can be raised. On the other hand, it does not have an adverse effect on the flavor of wine by work of a bacteria smell or a contamination enzyme. It can use not only for grape juice but for the fruit juice of citrus from the size of substrate specificity to the rhamnosyl glycoside of natural flavonoid of this enzyme.

## TECHNICAL FIELD

[Field of the Invention] This invention relates to the microorganism which produces new alpha-L-rhamnosidase, its manufacturing method, and its enzyme.

## PRIOR ART

[Description of the Prior Art] Linalool (linalool), geraniol (geraniol) which are contained in a grape, Monoterpenes, such as Nellore (nerol), citronellol (citronelol), and the alpha-terpineol (terpineol), have contributed to the scent of a grape or wine greatly. Although such monoterpenes are contained also as fragrant educt, the many exist as a glycoside which does not have fragrance.

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#### EFFECT OF THE INVENTION

[Effect of the Invention]this invention enzyme has the activity which was dramatically excellent under high-concentration glucose and a high-concentration ethanol existence. It can use for brewing of wine.

That is, by using this invention enzyme with beta-D-glucosidase etc., a perfume component can be made to be able to increase in wine brewing, and the quality of wine can be raised. On the other hand, it does not have an adverse effect on the flavor of wine by work of a bacteria smell or a contamination enzyme. It can use not only for grape juice but for the fruit juice of citruses from the size of substrate specificity to the rhamnosyl glycoside of natural flavonoid of this enzyme.

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[Problem(s) to be Solved by the Invention]Therefore, the purpose of this invention is to provide the microorganism which produces new alpha-L-rhamnosidase provided with the characteristic which can be used for wine brewing, its manufacturing method, and its enzyme.

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## MEANS

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[Means for Solving the Problem] This invention persons ask for a microorganism which can produce alpha-L-rhamnosidase provided with the characteristic which can be used for wine brewing, As a result of searching centering on yeast with until now few examples of a report especially, one strain belonging to the Pichia (Pichia) group finds out producing new alpha-L-rhamnosidase, and came to complete this invention. Alpha-L-rhamnosidase activity is measured by a measuring method which crushes a biomass and for which a centrifugal supernatant liquid fraction is later mentioned after culture by a culture medium which specifically makes L-rhamnose the only carbon source for many microorganisms isolated from various kinds of sources of separation, and the target microorganism is chosen.

[0008] That is, according to this invention, alpha-L-rhamnosidase having the following character is provided.

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(2) Optimal pH : optimal pH at a time of using p-nitrophenyl alpha-L-rhamnopyranoside as a substrate and making it react for 10 minutes at 30 °C is in the 5.0 to 7.0 neighborhood.

(3) pH stability : when it holds for 30 minutes at 30 °C, enzyme activity stable in pH 5.0-7.0 is shown.

(4) Optimum temperature : optimum temperature at a time of using p-nitrophenyl alpha-L-rhamnopyranoside as a substrate and making it react for 10 minutes at 30 °C pH 6.5 is about 40 °C.

(5) Thermal stability : when it holds for 30 minutes in pH 6.5, if enzyme activity stable to 20 °C is shown and it is exceeded, it will be deactivated gradually.

[0009] (6) Inhibitor : mercury para-chlorobenzoate, mercuric chloride, and a cupric chloride receive inhibition, and it is hardly prevented in ethylenediaminetetraacetic acid and mercaptoethanol.

(7) Molecular weight : a molecular weight measured by an SDS-polyacrylamide-gel-electrophoresis method is about 88,000 dalton.

(8) Isoelectric point: An isoelectric point by isoelectric focusing is pI 4.9.

(9) Glucose tolerance : 76% of relative activity is shown in 500mM glucose.

(10) Ethanol resistance : 67% of relative activity is shown in 20 capacity % ethanol.

[0010] A manufacturing method of alpha-L-rhamnosidase of this invention is the method of acquiring alpha-L-rhamnosidase from culture medium obtained by belonging to the Pichia (Pichia) group and cultivating a microorganism which produces alpha-L-rhamnosidase.

[0011] Character of a separation strain to produce alpha-L-rhamnosidase of this invention is shown below.

A. Form a colony of morphometrical characteristic 1 opalescence.

2) Germination performs asexual growth.

3) Form 1 thru/or 4 ascospores of a bowler hat form by a glucose malt culture medium.

[0012]

B. physiological character 1 fermentability Glucose + galactose . - malt sugar - sucrose - trehalose . + melibiose - lactose -2 utilization nature . glucose + lactose - galactose . - raffinose - sorbose - ribitol . + ribose + mannitol + xylose + inositol - rhamnose +

methanol + sucrose + ethanol + malt sugar + nitrate + melibiose - (+utilization carries out -utilization does not carry out)

[0013]It refers to BARNETT & PAYNE YARROW YEASTS:Characteristics and identification (Second edition) based on the above mycology character, This strain was identified to *Pichia ANGUSUTA* (*Pichia angusta*). It was judged that it was a new strain from a point of producing above-mentioned new alpha-L-rhamnosidase. Therefore, it was named *Pichia ANGUSUTA* (*Pichia angusta*) X349, and a bacteria stock was deposited with National Institute of Bioscience and Human Technology. A deposition number is FERM P-17211. If a microorganism used for this invention is a strain which produces alpha-L-rhamnosidase of this invention by a microorganism belonging to a *Pichia* group besides said *Pichia ANGUSUTA* (*Pichia angusta*) X349, it will not interfere.

[0014]In this invention, it may be used for culture of the usual microorganism as a culture medium which produces alpha-L-rhamnosidase, and especially if a microorganism used grows, it will not be restricted. As a carbon source, although sucrose etc. can be used, for example, a point of producing a lot of alpha-L-rhamnosidase to especially L-rhamnose is desirable. As a nitrogen source, peptone is raised, for example. To a culture medium, mineral salt, for example, sodium chloride, besides a carbon source and a nitrogen source can be added. Although the culture temperature should just be a temperature which a microorganism to be used grows, near [ good ] 28 \*\* of growth is preferred. Initiation pH of a culture medium to be used has the 4.0 preferred neighborhoods. Alpha-L-rhamnosidase is accumulated into culture medium at culture time by [ for growth of the bacillus concerned, and alpha-L-rhamnosidase production / sufficient ] usually cultivating for 48 hours, although time continuation is carried out.

[0015]Biomasses are collected with filtration, centrifugal separation, or filtration after culture, and supernatant liquid produced by crushing a biomass by mechanical disruption processing of lytic enzyme processing or ultrasonication, an French press, a homogenizer, etc., and centrifuging is used as crude enzyme liquid. Although crude enzyme liquid can also be used as it is, separation refinement can be carried out according to the separation refinement methods of common enzymes, such as ammonium sulfate curing salting and a solvent sedimentation method. An enzyme preparation with high activity purity can also be prepared combining suitably a refining means of the usual enzymes, such as ion exchange chromatography and gel filtration chromatography. The character of this invention enzyme obtained by refining combining various chromatography methods from crude enzyme liquid is as follows. Measurement of enzyme activity was performed to below by a following method.

[0016][Enzyme activity measurement] 0.2 ml of 2mM p-nitrophenyl alpha-L-rhamnopyranoside solutions which dissolved in the buffer solution are mixed to 0.2 ml of enzyme sample solutions diluted with 50mM sodium phosphate buffer solution (pH 6.5), and it is made to react to them for 10 minutes at 30 \*\*. Then, 1.6 ml of 1M sodium carbonate is added, a reaction is stopped, and p-nitrophenol separated by measuring an absorbance at 405 nm is quantified. One enzyme unit (U) which shows enzyme activity was defined in the above-mentioned conditions, i.e., 30 \*\*, and pH 6.5 as an amount of enzymes which makes p-nitrophenol of 1micromol generate in 1 minute after p-nitrophenyl alpha-L-rhamnopyranoside.

[0017](1) OPERATION -- when p-nitrophenyl alpha-L-rhamnopyranoside is made to act as a substrate, separate p-nitrophenol.

Optimal pH : (2) p-nitrophenyl alpha-L-rhamnopyranoside is used as a substrate, In accordance with said enzyme activity measurement, relative activity was measured except using a 50mM citrate-phosphate buffer solution (pH 3.0-7.0), 50mM phosphate buffer solution (pH 8.0-9.0), and 50mM glycine sodium hydroxide buffer solution (pH 10.0) as buffer solution. The result is as being shown in drawing 1, and reaction optimal pH is in the 6.0 to 7.0 neighborhood.

pH stability : (3) A 50mM citrate-phosphate buffer solution (pH 3.0-7.0), After holding for 30 minutes at 30 \*\* in each buffer solution of 50mM phosphate buffer solution (pH 8.0-9.0) and 50mM glycine sodium hydroxide buffer solution (pH 10.0), residual activity was measured in accordance with said enzyme activity measurement. The result is as being shown in drawing 1, and a stable pH range is pH 5.0-7.0.

(4) Optimum temperature : in accordance with said enzyme activity measurement, relative activity was measured at various temperature. The result is as being shown in drawing 2, and optimum temperature is about 40 \*\*.

(5) Thermal stability : after holding for 30 minutes in 50mM phosphate buffer solution (pH 6.5), residual activity was measured in accordance with said enzyme activity measurement. As a result, if this enzyme shows enzyme activity stable to 20 \*\* and it is exceeded as shown in drawing 2, it will be deactivated gradually.

[0018](6) Influence of various reagents : influence on this enzyme of various reagents was investigated. That is, various reagents shown in Table 1 were dissolved so that it might become 50mM phosphate buffer solution (pH 6.5) with 1mM, an enzyme solution was mixed to this, residual enzyme activity after holding for 10 minutes in 30 \*\* was measured in accordance with said enzyme activity measurement, and a result of Table 1 was obtained.

[Table 1]

[0019](7) Molecular weight : it is SDS-polyacrylamide gel electrophoresis () about this refined enzyme. [ PhastSystem and ] The molecular weight which measured by performing PhastGel homogeneous12.5 and PhastGel SDS bufferstrips (Pharmacia manufacture) is about 88,000 dalton. To the standard of a molecular weight, b type phosphorylase (molecular weight of 94,000 dalton), Bovine serum albumin (molecular weight of 67,000 dalton), ovalbumin (molecular weight of 43,000 dalton), Carbonic anhydro RAZE (molecular weight of 30,000 dalton), soybean tpsin inhibitor (molecular weight of 20,100 dalton), and lactalbumin (molecular weight of 14,400 dalton) were used.

(8) Isoelectric point: The isoelectric point which measured about this refined enzyme by performing isoelectric focusing (PhastSystem, PhastGel IEF 3-9 (Pharmacia manufacture)) is pI4.9. To the standard of an isoelectric point, horse myoglobin (acidic:pI6.85), Homo sapiens carbonic anhydro RAZEB (pI6.55), cow carbonic anhydro RAZEB (pI5.85), the beta-lactoglobulin A (pI5.20), a soybean trypsin inhibitor (pI4.55), and amyloglucosidase (pI3.50) were used.

[0020](9) Glucose tolerance : in accordance with said enzyme activity measurement, relative activity was measured in 50mM phosphate buffer solution (pH 6.5) containing glucose of 0 - 500mM concentration. The result is as being shown in drawing 3, and shows 76% of relative activity also in 500mM glucose.

(10) Ethanol resistance : in accordance with said enzyme activity measurement, relative activity was measured in 50mM phosphate buffer solution (pH 6.5) containing the ethanol of 0 - 20 capacity % concentration. It is as being shown in drawing 4, and in 10 capacity % ethanol, the result shows 85% of relative activity, and shows 67% of relative

activity in 20 capacity % ethanol.

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## EXAMPLE

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[Example]Next, although the example of this invention is given and this invention is explained in detail, the example shown below is an example and does not limit the technical scope of this invention.

Example 1 : Culture of a strain, and 1.0% of production L-rhamnose of alpha-L-rhamnosidase, Peptone 0.5%, the culture medium which consists of 0.3% of a yeast extract and 0.3% of a malt extract was adjusted the pH to 4.0 with chloride, 100 ml of this was taught to each 40 Erlenmeyer flasks of 500-ml \*\*, and autoclaving was carried out for 15 minutes 120 \*\*. The Pichia ANGUSUTA (Pichia angusta)X349 share was inoculated into this, and it cultivated on the rotation agitator for two days at 28 \*\* and 220 rpm. The obtained culture medium 4L is centrifuged (for 1000xg and 10 minutes), biomasses are collected, and it is 20mM. A screw/tris buffers (pH 6.5) washed twice. This was suspended to the buffer solution, the 2.5-times the amount glass bead (0.45-0.55 mm in diameter) of biomass weight was added, and the biomass was crushed. Except for biomass debris, the cell extract was obtained by centrifugal separation (for 1000xg and 10 minutes). Ultracentrifuge processing (for 100,000xg and 90 minutes) of this was carried out, and it separated into the microsome fraction and the supernatant liquid fraction. Since all the activity existed in the supernatant liquid fraction when such fraction alpha-L-rhamnosidase activity was measured, the refining operation described below was presented with the supernatant liquid fraction.

[0022]Example 2: Especially refining operation shown below in refining of an enzyme was altogether performed at 4 \*\*, unless it refused.

(1) Ammonium sulfate precipitate refined 600 ml of supernatant liquid fractions obtained in ammonium sulfate precipitate example 1. the precipitate formed from saturation in the fraction of saturation 80% 30% -- centrifugal separation (1,000xg.) It carried out for 30 minutes, collected, dissolved in the 100mM sodium acetate buffer solution (pH 6.0) containing 1M NaCl, 1mM MnCl<sub>2</sub>, 1mM CaCl<sub>2</sub>, and 0.003mM NaN<sub>3</sub>, and dialyzed to the buffer solution.

(2) The enzyme solution which carried out concanavalin A-sepharose column chromatography dialysis was given to the concanavalin A-sepharose column (phi2.6x6.5cm) beforehand equilibrated with the buffer solution. Since the target enzyme did not stick to this column, it was eluted with the buffer solution. Activity fractions are collected, and it condenses with ultrafiltration equipment (made by PM-10:Amicon), and is 20mM. It dialyzed to a screw/tris buffers (pH 6.5).

(3) DEAE Bio-Gel A They are 20mM beforehand about the enzyme solution which carried out agarose column chromatography dialysis. The DEAE Bio-Gel A agarose column (phi2.6x6.0cm) equilibrated with a screw/tris buffers (pH 6.5) was given. After the buffer solution washed, it was eluted by the sodium chloride linear gradient (0-0.3M) using the buffer solution. Activity fractions were collected and it dialyzed to 50mM sodium acetate buffer solution (pH 5.0).

[0023](4) An arabinose sepharose 6B affinity column chromatography rhamnose sepharose 6B affinity carrier, In accordance with how to recommend a manufacturing company for the epoxy activation sepharose 6B (made by Pharmacia biotech AB), rhamnose was combined and it prepared. The enzyme liquid obtained by the DEAE Bio-GelA agarose column chromatography mentioned above, The rhamnose sepharose

6B affinity column (phi1.5x7.2cm) beforehand equilibrated with 50mM sodium acetate buffer solution (pH 5.0) was given, and the buffer solution washed. Next, it was eluted with the buffer solution containing 1M NaCl, activity fractions were collected, and it dialyzed to distilled water.

(5) The enzyme solution which carried out hydroxyapatite column chromatography dialysis, The hydroxyapatite column (phi1.5x2.8cm) beforehand equilibrated with 10mM sodium phosphate buffer solution was given, the buffer solution washed enough, the back was eluted by the sodium phosphate linear gradient (10 - 200mM), and activity fractions were collected.

[0024]The activity of the enzyme in every above purification process is shown in Table 2. Cow serum albumin is used for protein as a standard substance, and it is the bottom in fixed quantity by the BURADOFODO method (Bradfordmethod).

[Table 2]

rha-sepharose 6B: Rhamnose sepharose 6B[0025]Example 3: The decomposition examination of the various chromophoric substrates shown in Table 3 was done using the refining enzymes obtained in substrate specificity example 2. The decomposition examination was done according to the method of said enzyme activity measurement, and the existence of activity was investigated by generation of p-nitrophenol. A result is shown in Table 3.

[Table 3]

[0026]Although this enzyme acts on p-nitrophenyl alpha-L-rhamnopyranoside and p-nitrophenol is separated, It hardly acts on p-nitrophenyl alpha-L-arabino furanoside, p-nitrophenyl beta-D-glucopyranoside, p-nitrophenyl beta-D-xylopyranoside, and p-nitrophenyl beta-D-cello BIOSHIDO, but it turns out that it is an enzyme with high substrate specificity.

[0027]1000 ml of grape juice (muscat OBU Alexandria kind) adjusted the pH to 7.0 by operation sodium hydroxide to the glycosides of Example 4:grape juice origin -- a synthetic adsorption resin column (phi -- 22 mm x 350 mm) Amberlite XAD-2: It let it pass to made in loam and Haas. 500 ml of distilled water washed this, and water soluble compounds were removed. After removing through and free terpenes for 1000 ml of pentane-ether (1:1) furthermore, glycosides were made eluted with 1000 ml of methanol. Concentration hardening by drying of this fraction was carried out under 30 \*\* and decompression, and 100 mg of glycosides were obtained. 10 mg of the above-mentioned glycosides were dissolved in 2 ml (0.5U, 50mM sodium phosphate buffer solution (pH 6.5)) of refining enzyme solutions obtained in Example 2, and it was made to react for two days at 30 \*\*. After 5 ml of pentane-ether (1:1) having extracted the separated terpenes twice and condensing it, it analyzed on condition of the following using capillary gas chromatography. A result is shown in Table 4. Thus, the enzyme of this invention disassembles the glycosides of grape juice, and has the outstanding character to which a perfume component is made to increase notably.

[Table 4]

[0028](Analysis condition)

device: -- Hewlett Packard 5980 column: -- a DB-WAX capillary column (made by phi0.25-mm x 30 m:J&WScientific)

detector: -- FID helium flow \*\*: -- a part for 2.0-ml/-- temperature program: -- -> with a temperature up of 75 \*\* 220 \*\* (a part for 4 \*\*/), and 220 \*\* (15 minutes) of maintenance sample injection section temperature: -- 230 \*\* primary detecting element temperature: -- 220 \*\* internal standard: -- amount of 2-ethyl-1-hexanol samples: -- 2microL

[0029]Example 5 : Have a rhamnosyl group in the operation nonreducing terminal to the rhamnosyl glycoside of natural flavonoid. The decomposition product of naringin (naringin), rutin (rutin), hesperidin (hesperidin), and the rhamnosyl glycoside of quercitrin (quercitrin) was analyzed using silica gel thin layer chromatography. That is, the reaction solution in which 0.5 ml of solutions (0.5U, 50mM sodium phosphate buffer solution pH6.5) of the refining enzymes obtained in Example 2 were made to dissolve 5 mg of substrates was held at 30 °C overnight. This sample was given to 4.0µl and a silica gel thin layer (DC-Alufolien Kieselgel 60: made by Merck Co.). After developing using acetonitrile distilled water (5:1, V/V) mixture, using rhamnose and glucose as a standard substance, the ethanol solution of sulfuric acid was sprayed 10%, it heated for 5 minutes at 105 °C, and sugars were detected.

[0030]As a result, naringin (naringin), the rutin (rutin), and the rhamnosyl glycoside of hesperidin (hesperidin) received decomposition by alpha-L-rhamnosidase, and rhamnose was detected. On the other hand, rhamnose was not detected about the rhamnosyl glycoside of quercitrin (quercitrin).

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## DESCRIPTION OF DRAWINGS

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### [Brief Description of the Drawings]

[Drawing 1]It is a graph which shows the stable pH region and operation pH region of *Pichia ANGUSUTA* (*Pichia angusta*)X349 share of alpha-L-rhamnosidase to produce.

[Drawing 2]It is a graph which shows the stable temperature region and operative temperature region of *Pichia ANGUSUTA* (*Pichia angusta*)X349 share of alpha-L-rhamnosidase to produce.

[Drawing 3]It is a graph which shows the activity of the *Pichia ANGUSUTA* (*Pichia angusta*)X349 share alpha-L-rhamnosidase to produce, and the relation of glucose concentration.

[Drawing 4]It is a graph which shows the activity of the *Pichia ANGUSUTA* (*Pichia angusta*)X349 share alpha-L-rhamnosidase to produce, and the relation of ethanol concentration.

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## DRAWINGS

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[Drawing 1]

[Drawing 2]

[Drawing 3]

[Drawing 4]

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(54) 【発明の名称】  $\alpha$ -L-ラムノシダーゼおよびその製造方法

(57) 【要約】 (修正有)

【課題】 高濃度のグルコースおよびエタノール存在下においても活性低下の少ない、グルコース耐性およびエタノール耐性を備えた $\alpha$ -L-ラムノシダーゼおよびその製造方法の提供。

【解決手段】 ピシア属に属する微生物が生産する、以下の理化学的性質を有する $\alpha$ -L-ラムノシダーゼ：

(1) 作用：p-ニトロフェニル- $\alpha$ -L-ラムノピラノシドを基質として作用させた場合、p-ニトロフェノールを遊離する。(2) pH安定性：30℃にて30分間保持した場合、pH5.0～7.0で安定した酵素活性を示す。(3) 至適温度：p-ニトロフェニル- $\alpha$ -L-ラムノピラノシドを基質としてpH6.5で30℃で10分間反応させたときの至適温度は約40℃である。(4) エタノール耐性：20容量%エタノール中において67%の相対活性を示す。



## 【特許請求の範囲】

【請求項1】 下記の性質を有する $\alpha$ -L-ラムノシダーゼ。

(1) 作用：p-ニトロフェニル- $\alpha$ -L-ラムノピラノシドを基質として作用させた場合、p-ニトロフェニルを遊離する。

(2) 至適pH：p-ニトロフェニル- $\alpha$ -L-ラムノピラノシドを基質として30℃で10分間反応させたときの至適pHは6.0～7.0付近にある。

(3) pH安定性：30℃にて30分間保持した場合、10 pH5.0～7.0で安定した酵素活性を示す。

(4) 至適温度：p-ニトロフェニル- $\alpha$ -L-ラムノピラノシドを基質としてpH6.5で30℃で10分間反応させたときの至適温度は約40℃である。

(5) 熱安定性：pH6.5にて30分間保持したとき、20℃まで安定した酵素活性を示し、それを超えるとき次第に失活する。

(6) 阻害剤：p-クロロ安息香酸水銀、塩化第二水銀、塩化第二銅により阻害を受け、エチレンジアミン四酢酸、メルカプトエタノールでは殆ど阻害されない。20

(7) 分子量：SDS-ポリアクリルアミドゲル電気泳動法により測定した分子量は約88,000ダルトンである。

(8) 等電点：等電点電気泳動法による等電点はpI4.9である。

(9) グルコース耐性：500mMグルコース中において76%の相対活性を示す。

(10) エタノール耐性：20容量%エタノール中において67%の相対活性を示す。

【請求項2】 ピシア (*Pichia*) 属に属する微生物を培養して得られる培養物から請求項1に記載の $\alpha$ -L-ラムノシダーゼを採取することを特徴とする $\alpha$ -L-ラムノシダーゼの製造方法。30

【請求項3】 ピシア (*Pichia*) 属に属する微生物をラムノース (*rhamnose*) を含む培地で培養して得られる培養物から請求項1に記載の $\alpha$ -L-ラムノシダーゼを採取することを特徴とする $\alpha$ -L-ラムノシダーゼの製造方法。

【請求項4】 請求項1に記載の $\alpha$ -L-ラムノシダーゼ産生能を有するピシア・アングスタ (*Pichia angusta*) X349株 (FERM P-17211)。40

## 【発明の詳細な説明】

## 【0001】

【発明の属する技術分野】 本発明は新規な $\alpha$ -L-ラムノシダーゼ、その製造方法およびその酵素を生産する微生物に関する。

## 【0002】

【従来の技術】 ブドウに含まれる、リナロール (*linalool*)、ゲラニオール (*geraniol*)、ネ50

ロール (*nerol*)、シトロネロール (*citronellol*)、 $\alpha$ -テルピネオール (*terpineol*) 等のモノテルペン類は、ブドウやワインの香りに大きく寄与している。これらのモノテルペン類は、芳香性の遊離体としても含まれているが、その多くは、芳香性を有しない配糖体として存在している。

【0003】 これらの配糖体の多くは、6-O- $\alpha$ -L-アラビノフラノシル- $\beta$ -D-グルコピラノシド (6-O- $\alpha$ -L-arabinofuranosyl- $\beta$ -D-glucopyranosides)、6-O- $\alpha$ -L-ラムノピラノシル- $\beta$ -D-グルコピラノシド (6-O- $\alpha$ -L-rhamnopyranosyl- $\beta$ -D-glucopyranosides)、6-O- $\beta$ -D-アピオフラノシル- $\beta$ -D-グルコピラノシド (6-O- $\beta$ -D-apiofuranosyl- $\beta$ -D-glucopyranosides) といったジグリコシド類であることが知られている。

【0004】 これらのジグリコシド類を酵素的に加水分解反応すると、芳香性のモノテルペン類が遊離してくる。この加水分解反応は二段階で行われる。すなわち、まず、L-ラムノシダーゼ ( $\alpha$ -L-rhamnosidase)、 $\alpha$ -L-アラビノフラノシダーゼ ( $\alpha$ -L-arabinofuranosidase)、 $\beta$ -アピオシダーゼ ( $\beta$ -apiosidase) などによりグリコシド結合が切断され、それぞれ対応するモノテルペニル- $\beta$ -D-グルコシド (*monoterpenyl- $\beta$ -D-glucosides*) を生じる。次いで、このモノテルペニル- $\beta$ -D-グルコシドに $\beta$ -D-グルコシダーゼ ( $\beta$ -D-glucosidase) が作用する。第一段階の反応を触媒する加水分解酵素のうち、 $\alpha$ -L-ラムノシダーゼは、 $\alpha$ -L-ラムノース (*L-rhamnose*) を含むポリサッカライドの非還元末端の $\alpha$ -L-ラムノピラノシド残基を加水分解する酵素である。従って、 $\alpha$ -L-ラムノシダーゼを、 $\beta$ -D-グルコシダーゼなどと共に使用することにより、ワイン醸造において芳香成分を増加させて、ワインの品質を向上させることができる。

【0005】 しかしながら、ワイン醸造にこれらの酵素を利用するにあたっては、多くの克服すべき問題がある。一つには、グルコースあるいはエタノールの存在下でも酵素活性を有することが必要であることである。また、一つには、バクテリア由来の酵素の場合、好まれざるバクテリア臭があること、カビ由来の菌体外分泌型の粗酵素では、他の触媒活性のために、好まれざる副反応が起こるという問題がある。これらバクテリアやカビ由来の $\alpha$ -L-ラムノシダーゼについては報告があるが、一方、酵母由来のものについての報告はない。厳密に言えば、サッカロミセス・セレビジエ (*Saccharomyces cerevisiae*) 由来のものが報告されているが、その主たる触媒活性は $\beta$ -D-グルコシ

ダーゼであって、わずかに $\alpha$ -L-ラムノシダーゼ活性も有するというものにすぎない。

#### 【0006】

【発明が解決しようとする課題】従って、本発明の目的は、ワイン醸造に利用できる特性を備えた新規な $\alpha$ -L-ラムノシダーゼ、その製造方法およびその酵素を生産する微生物を提供することにある。

#### 【0007】

【課題を解決するための手段】本発明者らは、ワイン醸造に利用できる特性を備えた $\alpha$ -L-ラムノシダーゼを生産し得る微生物を求め、特に今まで報告例の少ない酵母を中心に探索を行なった結果、ピシア(Pichia)属に属する一菌株が新規な $\alpha$ -L-ラムノシダーゼを生産することを見出し、本発明を完成するに至った。具体的には、各種の分離源から単離した多くの微生物をL-ラムノースを唯一の炭素源とする培地で培養後、菌体を破碎し遠心清画分について、後述する測定法で $\alpha$ -L-ラムノシダーゼ活性を測定し、目的とする微生物を選択したものである。

【0008】すなわち、本発明によれば、下記の性質を有することを特徴とする $\alpha$ -L-ラムノシダーゼが提供される。

(1) 作用：p-ニトロフェニル- $\alpha$ -L-ラムノピラノシドを基質として作用させた場合、p-ニトロフェニールを遊離する。

(2) 至適pH：p-ニトロフェニル- $\alpha$ -L-ラムノピラノシドを基質として30℃で10分間反応させたときの至適pHは5.0～7.0付近にある。

(3) pH安定性：30℃にて30分間保持した場合、pH5.0～7.0で安定した酵素活性を示す。

(4) 至適温度：p-ニトロフェニル- $\alpha$ -L-ラムノ\*

#### B. 生理的性質

##### 1) 発酵性

|        |   |
|--------|---|
| グルコース  | + |
| ガラクトース | - |
| マルトース  | - |
| スクロース  | - |
| トレハロース | + |
| メリビオース | - |
| ラクトース  | - |

##### 2) 資化性

|        |   |                   |   |
|--------|---|-------------------|---|
| グルコース  | + | ラクトース             | - |
| ガラクトース | - | ラフィノース            | - |
| ソルボース  | - | リビトール             | + |
| リボース   | + | マンニトール            | + |
| キシロース  | + | イノシトール            | - |
| ラムノース  | + | メタノール             | + |
| スクロース  | + | エタノール             | + |
| マルトース  | + | 硝酸塩               | + |
| メリビオース | - | (+:資化する, -:資化しない) |   |

\*ピラノシドを基質としてpH6.5で30℃で10分間反応させたときの至適温度は約40℃である。

(5) 熱安定性：pH6.5にて30分間保持したとき、20℃まで安定した酵素活性を示し、それを超えると次第に失活する。

【0009】(6) 阻害剤：p-クロロ安息香酸水銀、塩化第二水銀、塩化第二銅により阻害を受け、エチレンジアミン四酢酸、メルカプトエタノールでは殆ど阻害されない。

(7) 分子量：SDS-ポリアクリルアミドゲル電気泳動法により測定した分子量は約88,000ダルトンである。

(8) 等電点：等電点電気泳動法による等電点はpI4.9である。

(9) グルコース耐性：500mMグルコース中において76%の相対活性を示す。

(10) エタノール耐性：20容量%エタノール中において67%の相対活性を示す。

【0010】また本発明の $\alpha$ -L-ラムノシダーゼの製造方法は、ピシア(Pichia)属に属し、 $\alpha$ -L-ラムノシダーゼを生産する微生物を培養し、得られた培養液から $\alpha$ -L-ラムノシダーゼを取得する方法である。

【0011】本発明の $\alpha$ -L-ラムノシダーゼを生産する分離菌株の性質について以下に示す。

#### A. 形態的性質

1) 乳白色のコロニーを形成する。

2) 出芽により無性増殖を行う。

3) グルコース麦芽培地で1ないし4個の山高帽形の子嚢胞子を形成する。

#### 【0012】

【0013】以上の菌学的性質をもとに BARNETT & PAYNE YARROW YEASTS: Characteristics and identification (Second edition) で検索し、この菌株をピシア・アングスタ (*Pichia angusta*) に同定した。また、上述の新規な $\alpha$ -ラーラムノシダーゼを生産する点から、新規菌株であると判断した。従って本菌株をピシア・アングスタ (*Pichia angusta*) X349 と命名し、工業技術院生命工学工業技術研究所に寄託した。寄託番号はFERM P-17211である。なお、本発明に用いる微生物は、前記ピシア・アングスタ (*Pichia angusta*) X349 の他、*Pichia* 属に属する微生物で本発明の $\alpha$ -ラーラムノシダーゼを生産する菌株であれば差し支えない。

【0014】本発明において、 $\alpha$ -ラーラムノシダーゼを生産する培地としては通常の微生物の培養に用いられるものでよく、使用される微生物が生育するものであれば特に制限されない。炭素源としては、例えばスクロースなどを用いることができるが、多量の $\alpha$ -ラーラムノシダーゼを生産する点からラーラムノースが特に望ましい。窒素源としては、例えば、ペプトンがあげられる。培地には炭素源、窒素源の他、無機塩例えば塩化ナトリウムを添加することができる。培養温度は使用する微生物が生育する温度であればよいが、生育の良好な28℃付近が好ましい。また使用する培地の初発pHは4.0付近が好ましい。培養時間は当該菌の生育と $\alpha$ -ラーラムノシダーゼ生産に十分な時間続行されるが、通常48時間培養することによって培養液中に $\alpha$ -ラーラムノシダーゼが蓄積される。

【0015】培養後、ろ過または遠心分離またはろ過法によって菌体を集め、溶菌酵素処理あるいは超音波処理、フレンチプレス、ホモジナイザーなどの機械的破碎処理により菌体を破碎し、遠心分離して得られた上清を粗酵素液として用いる。粗酵素液はそのまま使用することもできるが、硫酸塩析や溶媒沈殿法などの一般の酵素の分離精製法に準じて分離精製することができる。また、さらにイオン交換クロマトグラフィー、ゲルろ過クロマトグラフィーなどの通常の酵素の精製手段を適宜組み合わせることで活性純度の高い酵素標品を調製することもできる。粗酵素液から各種クロマトグラフィー法を組み合わせることで精製することにより得られた本発明酵素の性質は以下の通りである。なお、以下において、酵素活性の測定は次の方法により行った。

【0016】[酵素活性測定法] 50mMリン酸ナトリウム緩衝液 (pH6.5) で希釈した酵素サンプル溶液

0.2mlに、同緩衝液に溶解した2mM p-ニトロフェニル- $\alpha$ -ラーラムノピラノシド溶液0.2mlを混合し、30℃で10分間反応させる。その後、1M炭酸ナトリウム1.6mlを加えて反応を停止させ、405nmにおける吸光度を測定することにより遊離したp-ニトロフェノールを定量する。また酵素活性を示す1酵素単位 (U) は、上記条件、すなわち30℃、pH6.5において、p-ニトロフェニル- $\alpha$ -ラーラムノピラノシドから1分間に1 $\mu$ molのp-ニトロフェノールを生成させる酵素量として定義した。

【0017】(1) 作用: p-ニトロフェニル- $\alpha$ -ラーラムノピラノシドを基質として作用させた場合、p-ニトロフェノールを遊離する。

(2) 至適pH: p-ニトロフェニル- $\alpha$ -ラーラムノピラノシドを基質とし、緩衝液として50mMクエン酸-リン酸緩衝液 (pH3.0~7.0)、50mMリン酸緩衝液 (pH8.0~9.0)、50mMグリシン-水酸化ナトリウム緩衝液 (pH10.0) を用いる以外は前記酵素活性測定法に従い、相対活性を測定した。その結果は図1に示すとおりであり、反応至適pHは6.0~7.0付近にある。

(3) pH安定性: 50mMクエン酸-リン酸緩衝液 (pH3.0~7.0)、50mMリン酸緩衝液 (pH8.0~9.0)、50mMグリシン-水酸化ナトリウム緩衝液 (pH10.0) の各緩衝液中で30℃にて30分間保持した後、前記酵素活性測定法に従い、残存活性を測定した。その結果は図1に示すとおりであり、安定pH範囲は、pH5.0~7.0である。

(4) 至適温度: 前記酵素活性測定法に従い、各種温度において相対活性を測定した。その結果は図2に示すとおりであり、至適温度は約40℃である。

(5) 熱安定性: 50mMリン酸緩衝液 (pH6.5) 中で30分間保持した後、前記酵素活性測定法に従い、残存活性を測定した。その結果、本酵素は図2に示すように、20℃まで安定した酵素活性を示し、それを超えると次第に失活する。

【0018】(6) 各種試薬の影響: 各種試薬の本酵素に対する影響を調べた。すなわち、表1に示す各種試薬を50mMリン酸緩衝液 (pH6.5) に1mMとなるように溶解し、これに酵素溶液を混合し、30℃において10分間保持した後の残存酵素活性を前記酵素活性測定法に従って測定し表1の結果を得た。

【表1】

表1. ラムノシダーゼに対する各種試薬の影響

| 添加化合物 (1mM)       | 相対活性 (%) |
|-------------------|----------|
| 無添加               | 100      |
| CuCl <sub>2</sub> | 10.9     |
| CaCl <sub>2</sub> | 98.1     |
| ZnCl <sub>2</sub> | 94.3     |
| FeCl <sub>2</sub> | 100.0    |
| MgCl <sub>2</sub> | 99.0     |
| CoCl <sub>2</sub> | 95.2     |
| MnCl <sub>2</sub> | 101.9    |
| AlCl <sub>3</sub> | 102.9    |
| HgCl <sub>2</sub> | 6.2      |
| EDTA              | 98.1     |
| メルカプトエタノール        | 97.6     |
| p-クロロ安息香酸水銀       | 2.4      |

【0019】(7) 分子量：精製した本酵素について SDS-ポリアクリルアミドゲル電気泳動 (Phast System、PhastGel homogeneous 12.5、PhastGel SDS buffer strips (ファルマシア社製)) を行い、測定した分子量は約88,000ダルトンである。分子量の標準には、b型ホスホリラーゼ (分子量94,000ダルトン)、ウシ血清アルブミン (分子量67,000ダルトン)、卵白アルブミン (分子量43,000ダルトン)、カルボニックアンヒドローラーゼ (分子量30,000ダルトン)、ダイズトリプシンインヒビター (分子量20,100ダルトン) およびラクトアルブミン (分子量14,400ダルトン) を用いた。

(8) 等電点：精製した本酵素について等電点電気泳動 (Phast System、PhastGel IEF 3-9 (ファルマシア社製)) を行い、測定した等電点は、pI 4.9である。等電点の標準には、馬ミオグロビン (acidic: pI 6.85)、ヒトカルボニックアンヒドローラーゼB (pI 6.55)、牛カルボニックアンヒドローラーゼB (pI 5.85)、β-ラクトグロブリンA (pI 5.20)、大豆トリプシンインヒビター (pI 4.55)、アミログルコシダーゼ (pI 3.50) を用いた。

【0020】(9) グルコース耐性：0~500mM濃度のグルコースを含む50mMリン酸緩衝液 (pH 6.5) 中で、前記酵素活性測定法に従い、相対活性を測定した。その結果は図3に示すとおりであり、500mMグルコースにおいても76%の相対活性を示す。

(10) エタノール耐性：0~20容量%濃度のエタノールを含む50mMリン酸緩衝液 (pH 6.5) 中で、前記酵素活性測定法に従い、相対活性を測定した。その結果は図4に示すとおりであり、10容量%エタノール

中において85%の相対活性を示し、20容量%エタノール中においても67%の相対活性を示す。

#### 【0021】

【実施例】次に本発明の実施例を挙げて本発明を詳しく説明するが、以下に示す実施例は一例であって本発明の技術的範囲を限定するものではない。

実施例1：菌株の培養およびα-ラーラムノシダーゼの生産

ラーラムノース1.0%、ペプトン0.5%、酵母エキス0.3%、麦芽エキス0.3%からなる培地を塩酸でpH4.0に調整し、これを500ml容の三角フラスコ40本に100mlずつ仕込み、120℃、15分間加圧滅菌した。これにピシア・アングスタ (*Pichia angusta*) X349株を接種し、28℃、220rpmにて2日間回転攪拌機上で培養した。得られた培養液4Lを遠心分離 (1000×g、10分間) して菌体を集め、20mM ビス/トリス緩衝液 (pH 6.5) で2回洗浄した。これを同緩衝液に懸濁し、菌体重量の2.5倍量のガラスビーズ (直径0.45~0.55mm) を加え、菌体を破碎した。遠心分離 (1000×g、10分間) により菌体破砕物を除き、細胞抽出液を得た。これを超遠心処理 (100,000×g、90分間) してミクロゾーム画分と上清画分に分離した。これらの画分α-ラーラムノシダーゼ活性を測定すると、すべての活性が上清画分に存在していたので上清画分を以下に述べる精製操作に供した。

#### 【0022】実施例2：酵素の精製

以下に示す精製操作は、特に断らない限り、全て4℃にて行った。

##### (1) 硫酸アンモニウム沈殿

実施例1で得た上清画分600mlを硫酸アンモニウム沈殿により精製した。30%飽和から80%飽和の画分

にて生じた沈殿を遠心分離（1,000×g、30分間）して集め、1M NaCl、1mM MnCl<sub>2</sub>、1mM CaCl<sub>2</sub>および0.003mM NaN<sub>3</sub>を含む100mM酢酸ナトリウム緩衝液（pH6.0）に溶解し、同緩衝液に対して透析した。

（2）コンカナバリンA-セファロースカラムクロマトグラフィー

透析した酵素溶液を予め同緩衝液で平衡化したコンカナバリンA-セファロースカラム（φ2.6×6.5cm）に付した。目的の酵素は、本カラムに吸着しないので、同緩衝液で溶出した。活性画分を集め、限外ろ過装置（PM-10：Amicon社製）にて濃縮し、20mMビス／トリス緩衝液（pH6.5）に対して透析した。

（3）DEAE Bio-Gel A アガロースカラムクロマトグラフィー

透析した酵素溶液を予め20mMビス／トリス緩衝液（pH6.5）で平衡化したDEAE Bio-Gel A アガロースカラム（φ2.6×6.0cm）に付した。同緩衝液で洗浄した後、同緩衝液を用いた塩化ナトリウムリニアグラジエント（0～0.3M）で溶出した。活性画分を集め、50mM酢酸ナトリウム緩衝液（pH5.0）に対して透析した。

【0023】（4）アラビノース-セファロース6Bア\*  
表2. ラムノシダーゼの精製

| 精製工程           | 蛋白量<br>(mg) | 活性<br>(U) | 比活性<br>(U/mg) | 回収率<br>(%) | 精製度<br>(倍) |
|----------------|-------------|-----------|---------------|------------|------------|
| 粗抽出液           | 562.38      | 383.5     | 0.682         | 100.0      | 1.0        |
| 硫酸アンモニウム沈殿     | 286.46      | 232.3     | 0.811         | 60.6       | 1.2        |
| ConA-セファロース    | 73.28       | 148.5     | 2.026         | 38.7       | 3.0        |
| DEAE Bio-Gel A | 8.92        | 121.5     | 13.624        | 31.7       | 20.0       |
| rha-セファロース6B   | 1.65        | 40.8      | 24.727        | 10.6       | 36.3       |
| ヒドロキシアパタイト     | 0.28        | 9.5       | 33.929        | 2.5        | 49.7       |

rha-セファロース6B：ラムノース-セファロース6B

【0025】実施例3：基質特異性

実施例2で得られた精製酵素を用い、表3に示す各種発色基質の分解試験を行なった。なお、分解試験は前記酵素活性測定の方法に準じて行ない、p-ニトロフェノー

\* フィニティーカラムクロマトグラフィー

ラムノース-セファロース6Bアフィニティー担体は、エポキシ活性化セファロース6B（ファルマシア・パイオテックAB社製）を製造会社の推奨する方法に従い、ラムノースを結合させ、調製した。前述したDEAE Bio-Gel A アガロースカラムクロマトグラフィーにより得られた酵素液を、予め50mM酢酸ナトリウム緩衝液（pH5.0）で平衡化したラムノース-セファロース6Bアフィニティーカラム（φ1.5×7.2cm）に付し、同緩衝液で洗浄した。次に1M NaClを含む同緩衝液で溶出し、活性画分を集め、蒸留水に対して透析した。

（5）ヒドロキシアパタイトカラムクロマトグラフィー  
透析した酵素溶液を、予め10mMリン酸ナトリウム緩衝液で平衡化したヒドロキシアパタイトカラム（φ1.5×2.8cm）に付し、同緩衝液で十分洗浄した後、リン酸ナトリウムリニアグラジエント（10～200mM）で溶出し、活性画分を集めた。

【0024】以上の精製工程毎における酵素の活性を表2に示す。なお、タンパク質は、牛血清アルブミンを標準物質として用い、ブラドフォード法（Bradford method）により定量した。

【表2】

ルの生成により活性の有無を調べた。結果を表3に示す。

【表3】

表3. ラムノシダーゼの基質特異性

| 基 質                              | 相対活性 (%) |
|----------------------------------|----------|
| p-ニトロフェニル- $\alpha$ -L-ラムノピラノシド  | 100      |
| p-ニトロフェニル- $\alpha$ -L-アラビノフラノシド | 6.1      |
| p-ニトロフェニル- $\beta$ -D-グルコピラノシド   | 2.7      |
| p-ニトロフェニル- $\beta$ -D-キシロピラノシド   | 0.2      |
| p-ニトロフェニル- $\beta$ -D-セロピオシド     | 0.1      |

【0026】本酵素はp-ニトロフェニル- $\alpha$ -L-ラムノピラノシドに作用し、p-ニトロフェノールを遊離するが、p-ニトロフェニル- $\alpha$ -L-アラビノフラノシド、p-ニトロフェニル- $\beta$ -D-グルコピラノシド、p-ニトロフェニル- $\beta$ -D-キシロピラノシドおよびp-ニトロフェニル- $\beta$ -D-セロピオシドにはほとんど作用せず、基質特異性の高い酵素であることがわかる。

【0027】実施例4：ブドウ果汁由来のグリコシド類に対する作用

水酸化ナトリウムでpH7.0に調整したブドウ果汁（マスカット オブ アレキサンドリア種）1000mlを合成吸着樹脂カラム（ $\phi$ 22mm $\times$ 350mm、Amberlite XAD-2：ローム・アンド・ハース社製）に通した。これを蒸留水500mlで洗浄し、\*

\* 水溶性化合物を除去した。さらにペンタン-エーテル（1：1）1000mlを通し、フリーのテルペン類を除去した後、メタノール1000mlにてグリコシド類を溶出させた。この画分を30℃、減圧下にて濃縮乾固し、グリコシド類100mgを得た。実施例2で得られた精製酵素溶液2ml（0.5U、50mMリン酸ナトリウム緩衝液（pH6.5））に上記グリコシド類10mgを溶解し、30℃にて2日間反応させた。遊離したテルペン類を5mlのペンタン-エーテル（1：1）で2回抽出し、それを濃縮した後、キャピラリーガスクロマトグラフィーを用い下記の条件にて分析した。結果を表4に示す。このように本発明の酵素は、ブドウ果汁のグリコシド類を分解し、芳香成分を顕著に増加させる優れた性質を有する。

表4. ラムノシダーゼによるグリコシド類からのテルペン類の遊離

| テルペン類             | ブドウ果汁由来グリコシド類1mg当り遊離量 (ng) |        |
|-------------------|----------------------------|--------|
|                   | 未処理                        | 酵素処理   |
| リナロール             | 0.0                        | 1180.5 |
| $\alpha$ -テルピネオール | 0.0                        | 73.5   |
| シトロネロール           | 0.0                        | 63.5   |
| ネロール              | 0.0                        | 216.5  |
| ゲラニオール            | 0.0                        | 1075.0 |

【0028】（分析条件）

装置：Hewlett Packard 5980

カラム：DB-WAXキャピラリーカラム（ $\phi$ 0.25mm $\times$ 30m：J&W Scientific社製）

検出器：FID

ヘリウム流速：2.0ml/分

温度プログラム：昇温75℃ $\rightarrow$ 220℃（4℃/分）、保持220℃（15分）

試料注入部温度：230℃

検出部温度：220℃

内部標準：2-エチル-1-ヘキサノール

サンプル量：2 $\mu$ L

【0029】実施例5：天然のフラボノイドのラムノシルグリコシドに対する作用

非還元末端にラムノシル基を有する、ナリンジン（naringin）、ルチン（rutin）、ヘスペリジン（hesperidin）およびクエルシトリン（quercitrin）のラムノシルグリコシドの分解生成物をシリカゲル薄層クロマトグラフィーを用いて分析した。すなわち、実施例2で得られた精製酵素の溶液（0.5U、50mMリン酸ナトリウム緩衝液pH6.5）0.5mlに基質を5mg溶解させた反応溶液を30℃で一晩保持した。この試料を4.0 $\mu$ L、シリカゲル薄層（DC-Alufolien Kieselgel 60：メルク社製）に付した。ラムノースおよびグルコースを標準物質として用い、アセトニトリル-蒸留水（5：1，V/V）混液を用いて展開した後、10%硫酸のエタノール溶液を噴霧し、105℃で5分間加熱

して糖類を検出した。

【0030】その結果、ナリンジン (narigin)、ルチン (rutin) およびヘスペリジン (hesperidin) のラムノシルグリコシドは $\alpha$ -L-ラムノシダーゼによって分解を受け、ラムノースが検出された。一方、クエルシトリン (quercitrin) のラムノシルグリコシドについてはラムノースは検出されなかった。

【0031】

【発明の効果】本発明酵素は、高濃度のグルコースおよび高濃度のエタノール存在下においても非常に優れた活性を有しており、ワインの醸造に利用できる。すなわち、本発明酵素を $\beta$ -D-グルコシダーゼなどと共に使用することにより、ワイン醸造において芳香成分を増加させて、ワインの品質を向上させることができる。一方、バクテリア臭や夾雑酵素の働きによりワインの風味に悪影響を与えることもない。また、本酵素の、天然の\*

\*フラボノイドのラムノシルグリコシドに対する基質特異性の広さから、ブドウ果汁のみならず柑橘類の果汁にも利用することができる。

【図面の簡単な説明】

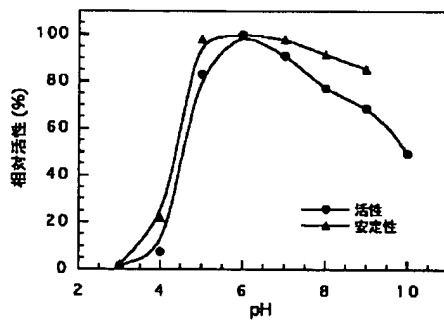
【図1】 ピシア・アングスタ (*Pichia angusta*) X349株の生産する $\alpha$ -L-ラムノシダーゼの安定pH域および作用pH域を示すグラフである。

【図2】 ピシア・アングスタ (*Pichia angusta*) X349株の生産する $\alpha$ -L-ラムノシダーゼの安定温度域および作用温度域を示すグラフである。

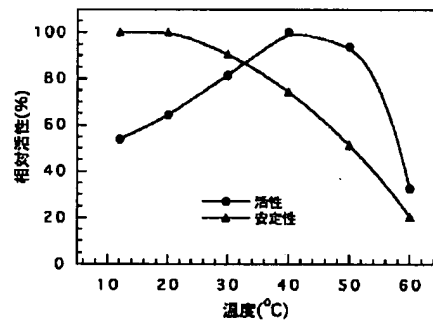
【図3】 ピシア・アングスタ (*Pichia angusta*) X349株の生産する $\alpha$ -L-ラムノシダーゼの活性とグルコース濃度の関係を示すグラフである。

【図4】 ピシア・アングスタ (*Pichia angusta*) X349株の生産する $\alpha$ -L-ラムノシダーゼの活性とエタノール濃度の関係を示すグラフである。

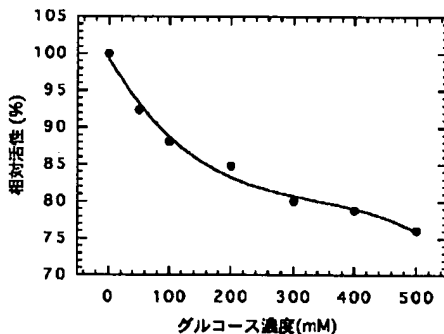
【図1】



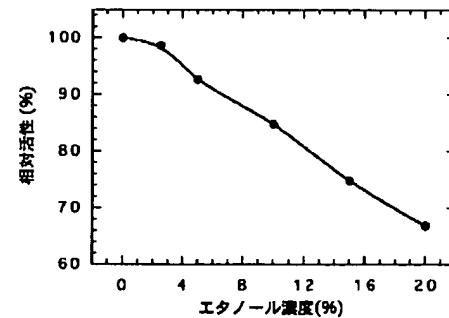
【図2】



【図3】



【図4】



フロントページの続き